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Results of the 2nd scientific workshop of the ECCO (III): basic mechanisms of intestinal healing

Rieder, Florian ; Karrasch, Thomas ; Ben-Horin, Shomron ; Schirbel, Anja ; Ehehalt, Robert ; Wehkamp, Jan ; de Haar, Colin ; Velin, Dominique ; Latella, Giovanni ; Scaldaferri, Franco ; Rogler, Gerhard ; Higgins, Peter ; Sans, Miquel

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DOI: <https://doi.org/10.1016/j.crohns.2011.11.009>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-76221>

Journal Article

Accepted Version

Originally published at:

Rieder, Florian; Karrasch, Thomas; Ben-Horin, Shomron; Schirbel, Anja; Ehehalt, Robert; Wehkamp, Jan; de Haar, Colin; Velin, Dominique; Latella, Giovanni; Scaldaferri, Franco; Rogler, Gerhard; Higgins, Peter; Sans, Miquel (2012). Results of the 2nd scientific workshop of the ECCO (III): basic mechanisms of intestinal healing. *Journal of Crohn's colitis*, 6(3):373-385.

DOI: <https://doi.org/10.1016/j.crohns.2011.11.009>

**Results of the second scientific workshop of the European Crohn's and Colitis
Organization (ECCO) (III):**

Basic mechanisms of Intestinal Healing

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Short title: Second ECCO Scientific Workshop – Basic mechanisms

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Abstract

The second scientific workshop of the European Crohn's and Colitis Organization (ECCO) focused on the relevance of intestinal healing for the disease course of inflammatory bowel disease (IBD). The objective was to better understand basic mechanisms, markers for disease prediction, detection and monitoring of intestinal healing, impact of intestinal healing on the disease course of IBD as well as therapeutic strategies. The results of this workshop are presented in four separate manuscripts. This section describes basic mechanisms of intestinal healing, identifies open questions in the field and provides a framework for future studies.

Key words

Inflammatory bowel diseases, Crohn's disease, Ulcerative colitis, Fibrosis, Fistula, Genetic variants

Abbreviations

5-ASA	5-aminosalicylic acid
AZA	Azathioprine
bFGF	Basic fibroblast growth factor
CD	Crohn's disease
CXCR	Chemokine (CXC motif) receptor
DAMP	Damage associated molecular pattern molecules
DSS	Dextrane sodium sulfate
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transformation
FAK	Focal adhesion kinase
FOX	Forkhead box
FSP	Fibroblast specific protein
HIF	Hypoxia inducible factors
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule 1
IFN	Interferon
IFX	Infliximab
IGF	Insulin-like growth factor
IL	Interleukin
IP	Interferon gamma-induced protein
LFA-1	Lymphocyte function-associated antigen 1
MAPK	Mitogen activated protein kinase

MIF	Macrophage migration inhibiting factor
MMP	Matrix metalloproteinase
MUC	Mucin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Nucleotide-binding oligomerization domain containing
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern molecules
PI3K	Phosphoinositide-3 kinase
SLPI	Secretory leucocyte protease inhibitor
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TAK	Transforming growth factor-β activated kinase
TFF	Trefoil factors
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll like receptor
TNBS	Trinitro-benzene sulfonic acid
TNF	Tumor necrosis factor
UC	Ulcerative colitis
VCAM	Vascular cell adhesion molecule 1

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1. Introduction

The scientific committee of the European Crohn's and Colitis Organization (ECCO) has launched a scientific workshop in 2010 on the relevance of intestinal healing for the disease course of inflammatory bowel disease (IBD). The overall objective of this workshop was to better understand and explore the importance of intestinal healing in IBD. The outcome of this workshop is presented in four separate publications: Mechanisms of intestinal healing (basic science), measures and markers to detect, achieve, and monitor intestinal healing, impact of intestinal healing on the course of IBD (natural history), and therapeutic strategies to enhance intestinal healing (therapy). This manuscript summarizes current knowledge about basic mechanisms of intestinal healing and discusses several key issues that need to be addressed in future studies.

The achievement of mucosal healing is a critical endpoint in the treatment of patients with both ulcerative colitis (UC) and Crohn's disease (CD). Achievement of mucosal healing in patients with IBD carries the prospect of influencing the natural history of this disease by the prevention of complications, such as need for surgery or hospitalization rates. The understanding of basic mechanisms of wound generation and healing is crucial for the improvement of existing and development of future therapies. It is not only important to evaluate key mechanisms in mucosal injury but also to discuss early as well as late events in the intestinal wound healing response. Striking differences in wound healing between different IBD patients can be observed, which can be explained by differences in genetic or epigenetic factors, differences in the intestinal luminal components or distinct responses to drug therapies.

2. Definition of and players in intestinal wound generation in IBD

2.1 Definition of mucosal injury

The commonly used definition of mucosal injury generally refers to macroscopically visible mucosal lesions during endoscopy, and this definition will be used hereafter. However, this term, which is helpful for clinical purposes, should be distinguished from the immune-mechanistic concept of mucosal injury, as employed in the research arena. This immune-mechanistic definition is multi-layered and encompasses several strata: gap junction disruption at a molecular level, increased epithelial permeability at a sub-cellular level, epithelial apoptosis, infiltration of activated inflammatory and lymphocytic cells at the cellular level, villous and crypt architectural changes, granuloma formation and disruption of the muscularis layer at the microscopic tissue level, and finally – the creation of erosions and ulcers macroscopically visible by endoscopic examination.

Moreover, in the current terminology, it is not universally agreed upon whether an isolated inflammatory process (for instance lamina propria inflammatory cell infiltration) qualifies for a mucosal injury or should this term be reserved for epithelial disruption only. Furthermore, it remains to be defined if mucosal healing should denote only a process restricted to the mucosa or should it include also the healing of sub-mucosa and muscularis layers as well as neuronal and lymphangiogenic elements of the intestinal wall.

2.2 Key players in mucosal injury

Mucosal injury is likely initiated by a combination of endogenous and environmental factors. At first stage, it is believed that food-derived compounds, virus and bacterial-derived factors as well as host-derived factors may cause epithelial cell destruction leading to pathogen-associated molecular pattern molecule (PAMP) and damage associated molecular pattern molecule (DAMP)-dependent activation of innate and adaptive immunity. Damaged mucosa is initially infiltrated by diverse inflammatory cells consisting of neutrophils, eosinophils, mast cells, inflammatory monocytes, activated macrophages and dendritic cells. In parallel, specific adaptive immune responses towards the intestinal flora are generated leading to the later recruitment of activated B cells, CD4+ and CD8+ T cells to the inflamed mucosa.

A number of molecules have been implicated in mediating the epithelial damage in IBD, leading to discernable mucosal lesions, but often their actual function and mechanism of action remains unclear. These molecules include, but are not restricted to:

Tumor necrosis factor- α (TNF): TNF was shown to induce intestinal epithelial cell apoptosis (1, 2), an effect that is abrogated by the ErbB4 growth factor receptor (3). However, it was also demonstrated that TNF exerts anti-apoptotic effects mediated by ErbB2 and epidermal growth factor (EGF) receptor (4), and that increased TNF levels correlate with inactive disease and lack of mucosal injury (5). Therefore, the exact role of TNF in instigating mucosal damage remains to be determined.

Reactive oxygen and nitrogen species: These mostly include superoxide O_2^- and nitric oxide NO which have been implicated in induction and propagation of epithelial mucosal injury (6). Increased levels of inducible nitric oxide (NO) synthase (iNOS) were found in IBD. iNOS

knock-out mice were protected against dextrane sodium sulfate (DSS) colitis (7) and experienced ameliorated trinitro-benzene sulfonic acid (TNBS) colitis when housed under SPF conditions (8). Notwithstanding, these iNOS knock-out animals paradoxically suffered from exaggerated colitis when induced by TNBS in non-specific pathogen free conditions (9) or when colitis was induced by the acetic acid model (10).

Superoxide O_2^- has been similarly implicated in the etiogenesis of mucosal damage in IBD. High levels of the enzyme superoxide dismutase (SOD) that catalyses and eliminates O_2^- was documented in IBD mucosa (11). Lactobacillus expression of SOD ameliorated intestinal inflammation in interleukin (IL)-10 deficient mice (12) and in a rat TNBS colitis model (13). In contrast, SOD overexpressing mice, expected to have higher metabolic clearance of O_2^- , paradoxically exhibited more severe DSS-induced colitis compared to wild type mice (7). It remains unclear, if a certain threshold level of mucosal O_2^- is required for efficient clearance of invading bacteria.

Matrix Metalloproteinases (MMP): Alterations in extracellular matrix (ECM) remodelling brought upon by an imbalance of certain MMPs and/or their inhibitors (in particular TIMP-1) have been implicated in inducing intestinal lesions in IBD. MMP-2, MMP-14, and TIMP-1 were increased significantly in the ulcerated mucosa of IBD patients but only slightly elevated in inflamed non-ulcerated section of intestine (14). Moreover, MMP-1,2,3 and 9 were increased in inflamed mucosa of IBD patients compared to the unaffected mucosa (14, 15). Inhibition of MMP activity was shown to ameliorate TNBS colitis (16). One possible mechanism for the preservation of mucosal integrity is action through substance P induced colonocyte proliferation (17).

Leptin: Leptin was found to induce intestinal epithelial damage via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) (18). In contrast, NFkB activation (not by leptin) was essential for protection of intestinal epithelial cells from ischemia-induced apoptosis (2, 19) and probiotic-induced protection from colitis (20).

Hypoxia inducible factor (HIF): HIF was found protective against TNBS colitis by enhancing barrier function and epithelial integrity (21, 22), but pro-inflammatory in DSS colitis by a macrophage migration inhibitory factor (MIF) dependent mechanism (23). These contradictory results were attributed to differences in the models used, namely the robust T-cell response in TNBS colitis as opposed to DSS, as well as to distinct effects of different HIF family members.

While multiple molecules and complex mechanisms seem to be instrumental in initiation and propagation of mucosal injury, a fundamental question remains unresolved: Why do certain areas of the gut exhibit mucosal injury while others remain unaffected? In ulcerative colitis patients high levels of pro-inflammatory cytokines, such as interferon (IFN)- γ , TNF, IL-6, IL-15, IL-18, and IL-23, can be found in endoscopically affected and unaffected mucosa. Interestingly, mediators of direct mucosal injury (MMP-3, TIMP-1, iNOS, granzyme-B) were elevated only in the affected mucosa (24). In CD patients only Granzyme B was elevated in affected but not unaffected mucosa, whereas TIMP-1 and iNOS were upregulated independently of endoscopic involvement (24). In addition, cathepsins have been shown to contribute to mucosal damage (25, 26). Thus, the differentiation between visibly ulcerated mucosa and normal appearing yet inflamed mucosa may be partly accounted for by the progression of the inflammatory process with up-regulation and/or expression of tissue damage mediators. It seems clear, however, that

there are other yet undefined mechanisms at play in dictating the progression to visible mucosal damage.

2.3 Key messages and questions to address in the future

Key messages

- The endoscopic definition of mucosal injury needs to be separated from the immune mechanistic definition.
- Mucosal injury is initiated by a multitude of endogenous and environmental factors and involves molecules, such as tumor necrosis factor, reactive oxygen species, matrix metalloproteinases, leptin, cathepsins and hypoxia inducible factor

Questions to address in the future

- We need to determine, which definition of mucosal injury or healing correlates best with the subsequent clinical course. Is it only the endoscopically visible lesion or would microscopic or even molecular perturbation of mucosal elements prove to be better predictors?
- What are the specific food-derived compounds, virus and bacteria-derived factors and host-derived or environmental danger signals inducing and perpetuating epithelial cell destruction?
- What is the specific mode of action of the above named mediators in pathogenesis of mucosal injury and healing?
- What triggers the progression from inflamed yet visually preserved mucosa to an inflamed overtly ulcerated mucosa and vice versa.

3. Early events in healing

3.1 Definition of early intestinal healing

The gastrointestinal mucosa provides a critical barrier, separating a myriad of environmental antigens within the intestinal lumen from the immune system of the host organism (27). Thus, early gastrointestinal wounding can be defined as a breakdown of the mucosal barrier function, leading to the translocation of antigens to the mucosal lamina propria, which is followed by the induction of acute and chronic inflammatory responses. Consequently, early mucosal wound healing can be described as the reestablishment of the intestinal mucosal barrier function.

Within the complex context of early gastrointestinal wounding and wound healing, several mechanistic components can be separated. Rather than providing a comprehensive review that has been given elsewhere (28-30) we will concentrate our presentation on the contribution of the luminal mucus compartment, endogenous antimicrobial peptides, and the intestinal epithelial cell layer to the early events in intestinal wound healing.

3.2 Gastrointestinal mucus layer

For a long time it has been known that goblet cell depletion is a common phenomenon in IBD. Recent methodological advances allowed for the definition of sophisticated substructures within the gastrointestinal mucus layer (e.g. its phosphatidylcholine layer, lamellar bodies, mucin composition and glycosylation) (28, 31), which have been demonstrated to be of crucial importance for its barrier function (32-34). The mucous layer and the bacteria residing in this

compartment form a protective ‘living wallpaper’ against exogenous bacteria through colonization resistance. Once the gastrointestinal mucus layer is disrupted, bacteria can penetrate the mucus and get in direct contact with epithelial cells, endangering mucosal homeostasis (35, 36). It is however likely that not only early mucosal wounding but also wound healing starts at the level of the intestinal mucus layer. An intact mucus layer is critical to immediately fill the gaps in an injured epithelial layer, providing an initial ‘seal’ and thus preventing further damage (37, 38). Understanding the mechanisms how mucus is secreted, composed and modified and how these processes are altered in IBD, are therefore highly important for development of further therapeutic approaches (39).

3.3 Paneth cells and defensins

After disruption of the intestinal mucus and direct contact of the intestinal microbiota with the epithelial cell monolayer a second line of defense against bacterial invaders is of increasing importance: Paneth-cell- as well as enterocyte-derived defensins. Once secreted they become part of the intestinal mucus and their antimicrobial activity is crucial for the mucosal barrier function (40-42). Its disruption has been associated with the development of intestinal inflammation in mouse models as well as chronic IBD in humans (43-45). It is therefore likely that insufficient anti-bacterial activity in proximity to the epithelial cell monolayer leads to an impaired early wound healing response, and future efforts should target an early reestablishment of this compartment.

3.4 Intestinal epithelial repair

Intestinal mucosal lesions in IBD, encompassing a numerous cells within the intestinal epithelial cell monolayer, necessitate a rapid resealing mechanism initiated by cells adjacent to the wound edges, which dedifferentiate (epithelial-mesenchymal transition, EMT), migrate over the wound to close the gap and re-differentiate including the formation of tight junctions (46). Smaller lesions allow a local purse-string mechanism via myosin light chain kinase activation in the epithelial cells surrounding the defect, rapidly closing the gap (47, 48). Host-derived factors support these healing mechanisms, e.g. growth factors and cytokines (49-52). Additionally, luminal factors (both host-derived and generated in the luminal environment) support intestinal epithelial wound healing, including intestinal trefoil factors (TFF, highly protease-resistant peptides secreted by goblet cells or their equivalents), bile acids, short chain fatty acids, adenine nucleotides, trace elements and the intestinal luminal microbiota themselves (52-57).

On the molecular level, these mediators induce multiple signaling events within intestinal epithelial cells, including NF- κ B-, mitogen activated protein kinase (MAPK)p38-, transforming growth factor (TGF)- β activated kinase (TAK)1-, focal adhesion kinase (FAK)-activation via Smad2/3 and protein kinase Akt-activation via phosphoinositide-3 kinase (PI3K) and via ErbB4. Downstream these signaling events exert mostly anti-apoptotic, pro-proliferative as well as pro-migratory effects (3, 58-62). Remarkably, many of these signaling mechanisms can also be induced independently of external factors through simple mechanical wounding of an intestinal epithelial cell monolayer alone (63-66). However, the exact mechanisms leading to the activation of the various signaling pathways via mechanical wounding in enterocytes remain to be elucidated.

Of note, many of the signaling molecules activated during intestinal epithelial wound-healing play an important role during acute and chronic intestinal inflammation as well (67-70). As intestinal epithelial wound infliction and healing are almost invariably occurring in an inflammatory context, pro- and anti-inflammatory signaling pathways and molecular events during wound healing form a complex and mutually interactive network. Importantly, treatment regimens aimed at suppressing certain pro-inflammatory signaling have proven to be deleterious via suppression of wound healing responses, especially in the presence of intestinal damage (19, 71). Further dissecting the intricate signaling network modulating intestinal epithelial cell proliferation, migration and apoptosis in gastrointestinal homeostasis and disease will be a prerequisite for the development of new therapeutic means aimed at these processes.

Technically, it has proven challenging to imitate local intestinal epithelial wounding and wound healing in a controlled model system *in vivo*. Puncture wounds created by endoscopic techniques have been used (72), as has been mucosal ulcer generation via acetic acid saturated filter discs applied to the serosal side of the small bowel (60, 73). Recently, two-photon laser technology allowed the generation of wounds encompassing single intestinal epithelial cells (38). On the other hand, loss of epithelial cells is not a prerequisite of breaches of the intestinal epithelial barrier: Since the intestinal epithelial cell monolayer has to be selectively permeable to allow for nutrient, electrolyte and water absorption, its ‘sealing mechanism’ (namely tight junctions located apically between the enterocytes) is constantly replenished and modified. Thus, apart from macroscopically distinguishable breaches like for example the loss of epithelial cells, ‘molecular breaches’ in tight junction permeability could be important in early wound generation (comprehensive review given in (30, 74)).

3.5 Key messages and questions to address in the future

Key messages

- Early intestinal healing can be defined as the re-establishment of the mucosal barrier function.
- The gastrointestinal mucus layer is of critical importance for the repair and maintenance of the intestinal barrier function.
- Disruption of endogenous antimicrobial activity is linked to intestinal injury and likely to an impaired wound healing response.
- Molecules that induce intestinal epithelial restoration are also important in intestinal inflammation.

Questions to address in the future

- What is the molecular mucus composition and structure (e.g. role of glycosylation) and its differences in health versus IBD?
- How is the secretion of phosphatidylcholine and antimicrobial effectors within the intestinal mucus layer regulated and stabilized?
- What are the mechanisms behind the balanced and symbiotic relation of bacteria and its host in the intestinal mucus compartment?
- What is the nature of intestinal ‘barrier breaches’: molecular (tight junction permeability) versus macroscopic? Which pathways or cell types are responsible for sensing a breach in the intestinal barrier: NF- κ B, MAPKp38, intestinal epithelial cells, dendritic cells?

- How do injury and inflammation intersect to rapidly induce wound healing mechanisms following an intestinal ‘barrier breach’ (mucus production, enterocyte proliferation, migration, apoptosis and cytokine secretion)?
- We need to further refine the technical means to investigate early gastrointestinal wounding/wound healing *in vivo* to enable more detailed investigations (e.g. two-photon-microscopy, lipidomics, proteomics and endoscopic techniques being the most promising approaches to date).

4. Late events in healing

4.1 Chronic wound repair and disease complications

In contrast to the intensive investigation of the immunological mechanisms of the early phases of intestinal inflammation and repair, the pathophysiology of chronic mucosal wound healing and the late events of repair remain largely unexplored. This is unfortunate, because insufficient (abscess, fistula) or excessive wound healing (fibrostenosis) are the main indications for surgery in patients with CD (75, 76). However, this process does not seem to happen in all patients, as up to one quarter of patients with CD continue to have a purely inflammatory phenotype, even after 25 years of disease (77). In patients with UC, chronic wound healing can induce fibrotic changes, including structural changes (haustral loss, colonic shortening), and disordered motility. These findings make it likely that intestinal inflammation is an important initiating event, which can either be followed by normal restitution, or by pathologic fibrosis and/or fistula formation (76, 78). It remains unclear, which signals and pathways initiate chronic wound healing abnormalities

in late healing, rather than normal restitution and resolution. If normal restitution and pathologic healing after inflammation are distinct pathways, these could be separately targeted, allowing selective therapy for the wound healing abnormalities seen in IBD.

4.2 Extracellular matrix as a driver of inflammation and wound repair

It is apparent from study of other organs that undergo fibrosis (e.g. lung, liver, and kidney), that once fibrosis is established it becomes an independent and self-perpetuating process, without a necessity for ongoing inflammation to drive matrix deposition (79). This could be true for fibrotic and stricturing CD as well. Possible mechanisms include [1] the intestinal ECM acting as a binding partner or reservoir for pro-fibrotic tissue factors (80), [2] the increased stiffness of the tissue acting as a stimulus for mechanosensitive cells to deposit and crosslink additional extracellular matrix in case of stricture formation, [3] reduced ECM stiffness leading to reduced matrix deposition in the case of fistulae (81). ECM is not an inactive structure, but directly regulates the inflammatory response and the process of healing and fibrosis by focal adhesions with immune and non-immune cells, such as myofibroblasts (82).

4.3 Sources of myofibroblasts and mechanisms of their activation

Activated myofibroblasts, a key effector cell type in intestinal wound healing, can be derived not only from resident mesenchymal cells, but also from other cell populations, including epithelial and endothelial cells (by a process termed epithelial-/endothelial- mesenchymal transition), stellate cells, pericytes, local stem cells and bone-marrow-derived cells (fibrocytes) (79, 83, 84). However, almost nothing is known about the functional relevance, mechanisms and targets for

intervention. A multitude of molecules derived from essentially all cell types involved in IBD can activate myofibroblasts. In addition, microbial PAMPs or DAMPs, critical for sterile inflammation, could be involved (85, 86).

The field of intestinal mesenchymal cells lacks critical technical tools: We need to define specific markers for myofibroblasts, a cell type most critically involved in wound healing and restitution. Specific subsets of mesenchymal cells may be identified that are particularly important in wound repair. Thus far no promoter specific for intestinal mesenchymal cells/myofibroblasts, useful for the generation of cell type specific transgenic mice, has been identified. Markers, such as fibroblast specific protein (FSP)-1 and α -smooth muscle actin (SMA) can be helpful, but are also expressed in additional cell types in intestinal fibrosis other than fibroblasts/myofibroblasts.

4.4 Regulation of extracellular matrix turnover

The fine balance between MMPs and TIMPs appears to be disturbed in chronically impaired wound healing in IBD (14). It is unclear which specific MMPs and TIMPs are involved and how they are regulated in this process. Our current understanding of intestinal fibrosis assumes that the amount of accumulated fibrotic tissue damage is linked to the likelihood of formation of fistulae or strictures. Effective pharmacological modulation of the MMP/TIMP-system could be helpful in the reversal of accumulated tissue fibrosis or healing of already formed fistulae (87).

Limited animal models for intestinal fibrosis exist, though several new models have recently been introduced. Virtually no animal model exists that helps our understanding of fistula formation. The field is in need of additional factors/molecules able to modulate the wound

healing response, which may include microRNAs. Delivery systems designed to direct these molecules to the deep layers of the human intestine need to be developed to allow broad application in clinical studies. Ultimately, non-invasive means to monitor and measure intestinal fibrosis and fistula formation in animals and humans are critical to provide endpoints for developing and testing specific anti-fibrotic therapies.

4.5 Key messages and questions to address in the future

Key messages

- Chronic intestinal wound healing abnormalities can lead to complications, such as formation of strictures or fistulae.
- The extracellular matrix is an active player in intestinal inflammation and repair.
- Intestinal myofibroblasts are derived from a multitude of sources and can be activated by classical pro-inflammatory signals and growth factors but also by environmental and bacterial components.
- In chronically impaired wound healing a disturbance of the MMP/TIMP balance can be observed.

Questions to be addressed in the future

- Which factors determine the switch from a purely inflammatory disease course to a complicated disease course? Do these factors appear early or late in the disease process? What are the differences between early and late disease?
- Is there a ‘point of no return’ in stricture and fistula formation?
- What are the mechanisms of auto-propagation of intestinal fibrosis and fistula formation?

- How does the matrix itself actively contribute to the abnormalities in wound healing?
- What is the main source of myofibroblasts in intestinal wound healing?
- What are the specific molecular markers of myofibroblasts?
- What are the main mediators of myofibroblast activation? What is their functional relevance, what are the mechanisms and targets for intervention?
- What is the role of the environment in activation of intestinal mesenchymal cells (possibly mediated through PAMPs and DAMPs)?
- What are the sources of MMPs and TIMPs in intestinal inflammation? Does an imbalance of this system early in the disease cause later complications? Can MMPs and TIMPs be used for the therapy of existing fistulae and strictures?

5. Determinants of intestinal healing

The ability to regenerate the intestinal mucosa greatly varies among different IBD patients, as well as, in a given patient, over time. Such heterogeneity implies the existence of certain factors influencing the molecular mechanisms responsible for mucosal healing. On one hand, genetic factors, including both the genetic background of the patient and post-transcriptional and epigenetic modifications could influence mucosal healing. On the other hand, acquired, environmental or “luminal” factors, such as the bowel microbiota, its products, diet components or even drugs might also play a key role in that process.

5.1 Genetic variants in IBD

Influence of genetic factors on IBD susceptibility and phenotype: According to our present understanding, both CD and UC develop in genetically susceptible subjects. The fact that genetic factors play a key role in the development of IBD is beyond any doubt. Many lines of evidence, including a higher risk in first-degree relatives, studies in monozygotic and dizygotic twins, greater prevalence in certain ethnic groups, identification of specific disease susceptibility genes and even results from animal models point towards a key role of genetic factors in IBD. Similarly, the fact that both CD and UC are polygenic conditions is undisputed. Two recent collaborative studies of the International Consortium for the Study of IBD genetics (88, 89) have identified 71 single nucleotide polymorphisms (SNPs) independently associated with a higher risk to develop CD (88) and 47 SNPs predisposing to UC (89). A very interesting concept emerging from these studies is the fact that some SNPs are common to both entities, suggesting that these factors facilitate “intestinal inflammation” in general, whereas other SNPs only increase the risk of developing either CD or UC, which means that these genetic factors are “more specific”, influencing only a certain type of intestinal inflammation.

Compared to our quite comprehensive understanding of the genetic factors determining CD and UC susceptibility, much less is known about the influence of genetic factors on disease phenotype, development of IBD-related complications and response to IBD therapies. The currently strongest disease-modifying gene seems to be nucleotide-binding oligomerization domain containing (NOD)2. Their three main variants have been associated to increased ileal disease location, stenosing phenotype and risk of surgery (90, 91).

Influence of genetic factors on intestinal healing in IBD patients: Unfortunately, there are no studies specifically aimed at identifying which genetic factors are independently associated with

a better or worse mucosal healing response in IBD patients. In spite of this obvious limitation, we can speculate that the genetic factors influencing, on one hand, the development of intestinal inflammation and injury and, on the other hand, the mechanisms of wound healing and repair might play a relevant role. In the first group a large number of genes, encoding the expression of molecules critical in inflammation and innate immunity, such as the NOD, TLR, and TNF families might constitute good candidate genes to influence mucosal healing. In the second gene group, and among the molecules responsible to regulate the wide spectrum spanning from physiological wound healing to abnormal fibrogenesis, several metalloproteinases and their inhibitors, as well as other key regulatory molecules, such as TGF- β , basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF)-1, would be excellent candidates.

To date most efforts in the field of IBD genetics have been devoted to the identification DNA-related genetic factors (single nucleotide polymorphisms). However, the potential relevance of other types of genetic factors, such as copy number variants, the degree of gene expression (mRNA), the existence of inhibitory RNA sequences (microRNAs), as well as the influence of DNA methylation and other epigenetic changes has been recently recognized and seems likely to be able to account for part of the observed variation in the process of mucosal healing in IBD patients. An excellent example is the study recently published by Arijs et al (92): Gene expression was assessed at both blood and tissue levels and the authors were able to identify a mucosal gene signature consisting of a combination of 5 expressed genes. This model showed a high accuracy in predicting response to infliximab in UC patients, as determined by endoscopic and histological remission (92).

5.2 Impact of butyrate on intestinal healing

As stated above the intestinal luminal compartment, such as mucus, endogenous anti-microbial factors and the epithelial barrier are critical in early wound generation and healing in the intestine. In addition to this an innumerable variety of compounds is present in the free lumen of our intestines.

A major fraction of these luminal factors, with reported impact on intestinal healing and the symbiotic host-to-microorganism relationship are derived directly or indirectly from bacteria. This occurs via affecting a multitude of immune and non-immune cells of the lamina propria at any time during development and activation of the human immune system (30). One of the best-characterized bacterial products known to have beneficial effects on the intestinal healing response is butyrate. Butyrate arises during the fermentation of starch by members of the anaerobe *Clostridium* subphylum species, i.e. clusters IV (*Faecalibacterium prausnitzii*, *Butyricicoccus pullicaecorum*) and XIVa (*Butyrivirio fibrisolvens*) (93). Interestingly, both the depletion of these members and the occurrence of antibodies directed against their flagellins are linked to IBD (94). As no comprehensive overview on all bacterial factors involved can possibly be presented, butyrate will serve as our example for luminal factors determining intestinal wound healing. The anti-inflammatory effect of butyrate has been shown in various *in vitro* and *in vivo* systems providing a rationale for assessing its therapeutic potential (95-97). Butyrate can affect early and late events in intestinal healing.

In vitro stimulation of intestinal biopsies with butyrate increased mucus synthesis (98). In a human colonic goblet cell line stimulation with butyrate from the apical side of the cells led to an increase in the expression of MUC2, MUC3 and MUC5B the genes encoding mucins (99). Very

limited information is available concerning the effects of butyrate on the expression of antimicrobial peptides. Treatment of colon epithelial cell lines with butyrate induces upregulation of human cathelicidin (LL-37) mRNA expression (100), a lysosomal antibacterial peptide. This is mediated by recruitment of PU.1 to the cathelicidin antimicrobial peptide promoter, the gene encoding LL-37. Butyrate exhibits a well-established role as a major energy source for enterocytes, but is also able to stimulate genes that are important for epithelial integrity, e.g. by affecting histone acetylation and DNA methylation. Butyrate facilitates tight junction assembly (101) and reduces metabolic stress induced loss of epithelial integrity – a mechanism that led to protection from enhanced bacterial translocation (102).

Butyrate has been shown to reduce the inflammatory cytokine driven production of MMP-1 and MMP-3 in colonic myofibroblasts (103). Interestingly, butyrate also reduced the expression of cytokine-induced IFN-gamma-induced protein (IP-10) by intestinal myofibroblasts, a factor reported to mediate chronic inflammation by recruiting T-cells and monocytes (104). To the contrary, butyrate was shown to enhance cytokine-induced stromelysin-1 expression thereby contributing to the inflammatory response (105). More knowledge about functional consequence of exposure of myofibroblasts to butyrate is needed.

These diverse actions of butyrate on different phases of intestinal wound healing makes it likely that additional luminal factors like bacteria themselves or their released products have a considerable role in this process. These factors need to be explored with the prospect as potential future therapeutics.

5.3 Molecular action of drugs on intestinal healing

While exploration of the role of genetic, epigenetic or luminal components to wound healing is critical for the development of future therapeutic approaches it is also prudent to thoroughly understand the molecular actions of our current IBD drug repertoire. This can help optimize existing treatment protocols by enhancing their efficacy and reducing their side effects.

Compounds, administered through any route, can signal at different levels of the intestinal mucosa (106): Pre-epithelial (intestinal mucus, bacteria), epithelial, post-epithelial (mucosal immune and non-immune compartments, modulation of cytokines and growth factors) or in a fashion combining all the above. Although ample data is available on clinical outcomes of IBD therapy, information regarding mechanisms of action are scarce.

5-aminosalicylic acid/sulfasalazine: Treatment with 5-aminosalicylic acid (5-ASA) has been associated with an abrogation of NF κ B activation *in situ* (107), which went along with reversal of microscopic alterations in the IBD mucosa like the mixed inflammatory infiltrate in the lamina propria, crypt architectural abnormalities, basally located lymphoid aggregates, basal plasmacytosis, villiform surface epithelial configuration and Paneth cell metaplasia (108). 5-ASA lowered IL-1 β and leukotriene B4 release from cultured biopsy specimens from the inflamed colonic mucosa of patients with active inflammatory bowel disease (109). *In vitro* models show that sulfasalazine exerted a direct effect on intestinal lamina propria leukocytes and peripheral blood leukocytes of IBD patients and healthy subjects, by triggering a potent pro-apoptotic effect, an action that stood in contrast to 5-ASA (110). Mesalazine is affecting cell migration and proliferation of intestinal epithelial cell lines, two key processes in mucosal healing, an effect dependent on TGF- β (111). The combination of N-acetylcysteine plus 5-ASA induced mucosal

healing by suppressing cyclooxygenase-2 gene expression and prostaglandin E2 levels in a TNBS rat colitis model (112).

Corticosteroids: Corticosteroid-induced healing of colonic inflammation is associated with a reduction of NFkB in nuclear extracts derived from intestinal mucosal biopsies of IBD patients (113). Dexamethasone lowered the release of IL-1 β and leukotriene B4 from IBD derived mucosal biopsies (109). High concentrations of prednisolone and budesonide, in contrast to lower concentrations, had inhibitory effects on proliferation and restitution of intestinal epithelial cells (114). These results were partially confirmed by other groups, that showed a steroid-induced inhibition of intestinal epithelial cell migration and proliferation (115). Dexamethasone suppressed growth factor induced epithelial restitution by inhibiting prostaglandins using two cultured cell wound-resealing models (116).

Azathioprine and Cyclosporine A: Azathioprine (AZA)-driven mucosal healing was associated with a decrease of the inflammatory infiltrate in the ileal mucosa from CD patients (117). AZA profoundly inhibits intestinal epithelial cell growth by causing a G2 cell cycle arrest, inducing apoptosis and dose-dependently inhibiting proliferation (118). The frequency of intestinal mucosal TNF secreting cells is reduced in pediatric CD patients upon treatment with cyclosporin A but not with corticosteroids or enteral nutrition treatment, although no clear relation existed between histological healing and the frequency of TNF secreting cells (119).

Infliximab: IBD patients receiving the anti-TNF antibody infliximab (IFX) showed an improvement in their increased intestinal permeability (120). Treatment leads to lower global numbers of CD4+ and CD8+ T lymphocytes and monocytes (121) and of mucosal CD68, a

marker for monocytes/macrophages (122). IFX down-regulated mucosal expression of forkhead box (Fox)p3, a marker for T-regulatory cells, while increasing their circulating levels (123). The mucosal expression of gelatinase B and TNF is reduced and this effect that was linked to endoscopic and/or histologic improvement (122). IFX lowers the expression of the cell recruitment molecules intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and lymphocyte function-associated antigen 1-(LFA-1). *In vitro* studies have shown that infliximab reduces the T-cell secretion of IFN- γ , likely because its synthesis is dependent on TNF levels. (124, 125). IFX attenuated intestinal mucosal MMP-3 and -12 synthesis but elevated the expression of antimicrobial peptides (121, 126-128). Finally, infliximab treatment was associated with improvement in morphology and function of the epithelial organelles, mucus secretion and recovery of the chorionic components at transmission electron microscope analysis (129).

Probiotics: Probiotics can induce regulatory cytokines, including IL-10 and TGF- β , and suppress TNF, in the mucosa of patients with CD and pouchitis (130). VSL#3, a probiotic mixture of 8 lactic acid bacteria probiotic strains or E. coli Nissle 1917, induces IL-10 and downregulates IL-12p40 production by lamina propria dendritic cells in patients with UC, changes similar to patients who were treated with corticosteroids (130, 131). VSL#3 has been linked to the enhancement of the increased expression of costimulatory molecules like CD80 (B7-1), CD86 (B7-2) and CD40, and of MHC class II in dendritic cells (131). E coli Nissle 1917 ameliorated DSS-colitis, an effect likely mediated by toll like receptor (TLR)-2 and TLR-4 (132). The probiotic bacterium bacillus polyfermenticus has been shown to increase angiogenic properties of human intestinal microvascular endothelial cells in a NF-kappaB/IL-8/chemokine (CXC motif)

receptor (CXCR)2-dependent manner, suggesting that it may be clinically used to facilitate intestinal wound healing (133).

Antibiotics: Even though metronidazole and ciprofloxacin are the most commonly used antibiotics in IBD many studies were performed on rifaximin, a non-absorbable derivative of rifamycin. In the TNBS-colitis model rifaximin reduced colitis severity and decreased the levels of several pro-inflammatory cytokines (134). Ciprofloxacin inhibits the cytokine-induced iNOS mRNA expression in HT-29 cells and a similar inhibitory effect was detected in vitro in cultures of normal colonic tissue and in cultures of colonic tissue from ulcerative colitis patients (135).

5.4 Key messages and questions to address in the future

Key messages

- The link between genetic variants and susceptibility to IBD has been well established. However information on the influence of genetic factors on intestinal healing is lacking.
- Luminal factors, directly or indirectly derived from bacteria, have an impact on intestinal healing and could account for the variability in the wound healing response.
- The mechanisms of action of our current IBD drug repertoire are under investigation, but still poorly understood.

Questions to address in the future

- What is the influence of different genetic and epigenetic factors (SNPs, copy number variants, mRNA gene expression, microRNA expression) - alone or in combination - on mucosal healing?

- Trials with the primary outcome of intestinal healing need to be designed.
- Are local epigenetic changes involved in the focal and segmental nature of fibrosis and fistula formation?
- What are the key components of the luminal compartment that trigger intestinal inflammation and repair?
- How can the observed alterations in the luminal compartment during intestinal inflammation (e.g. butyrate) be used to guide us to novel therapeutic approaches?
- We need to develop cell or compartment specific drug delivery systems
- How do the currently available IBD therapeutics influence the late healing response and how do they affect the occurrence of complications on a molecular level?

Conflict of interest

The following authors have no conflict of interest: Florian Rieder, Thomas Karrasch, Anja Schirbel, Robert Ehehalt, Jan Wehkamp, Dominique Velin, Colin de Haar, Giovanni Latella, Franco Scaldaferri, Gerhard Rogler, Miquel Sans.

The following authors have conflict of interest: Shomron Ben-Horin (Abbott; Schering-Plough), Peter Higgins (JBR Pharma).

Acknowledgements

Andreas Sturm, Edouard Louis and Florian Rieder organized the workshop in behalf of the ECCO scientific committee. All authors wrote specific parts of the manuscript, critically reviewed the completed manuscript and read as well as approved the final version.

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